

Modified Growth Media of *Bacillus amyloliquefaciens* S20A1 and Biocontrol of Bacterial Leaf Blight of Rice

A.T. Cho and S. Kasem *

Department of Plant Pathology, Faculty of Agriculture, Kasetsart University Bangkok 10900, Thailand

* Corresponding author. E-mail: agrsupot@ku.ac.th

Received: 31 July 2018 Accepted: 15 January 2019

ABSTRACT

The effective and high cost of general culture media under laboratory conditions is a major challenge for mass production of biological control agents. Generally, beans (Leguminosae) are well-known crop with highly nutrient contents that could be supported the growth of bacteria. The study was conducted to develop the alternative low cost of media from locally available beans (black bean, mung bean and soybean) for enhance the capacity of the multiplication and biocontrol activities of *Bacillus amyloliquefaciens* S20A1, the effective antagonistic bacterial strain, to control bacterial leaf blight disease (BLB) of rice caused by *Xanthomonas oryzae* pv. *oryzae*. Maximum cell growth and percentage of BLB disease reduction was observed at two modified media formula, black bean + soybean mixture medium (BS) at the ratio of 1:1 and mung bean + soybean mixture medium (MS) (1:1 ratio) with 1.00×10^{10} CFU /mL and 63.10%, and 1.3×10^{10} CFU /mL and 63.82%, respectively when comparing with the general culture condition that showed 3.6×10^9 CFU/mL and 61.68% disease reduction. The composition of 30 grams mixed bean powder per liter of both modified media and then incubated on 150 rpm rotary shaker for 36 hours were the effective culture. The cost per liter of media was reduced 22 times and 19 times comparing with LB medium, readymade laboratory medium. Two bean powder extract media (BS and MS) also used for the cell multiplication and promotion of the biocontrol activities of fresh cell but also for formulated of strain S20A1 to control the bacterial leaf blight disease of rice. This study could provide reliable basis for small scale production and a scale-up to the industrial level.

Keywords: *Bacillus amyloliquefaciens*, bacterial population density, biocontrol mechanisms, culture media, carbon and nitrogen sources in beans, secondary metabolites

Thai J. Agric. Sci. (2018) Vol. 51(4): 195–207

INTRODUCTION

The bacterial leaf blight (BLB) of rice (*Oryza sativa* L.), caused by *Xanthomonas oryzae* pv. *oryzae* (Xoo), is the one of the serious problems with responsibility for severe economic losses in rice production (Xu *et al.*, 2015). Increasing the sustainable agriculture and safety farm production system, the use of effective microorganisms using as biological control has become more interesting. The important mechanisms by which antagonistic microorganisms

are generally attributed to parasitism, competition, and the production of secondary metabolites with antibiotics or exoenzymes effects on pathogens suppression, host resistance induction and plant growth promotion. The use of *Pseudomonas* and *Bacillus* strains have been reported for the biocontrol of rice pathogens such as Xoo, *Magnaporthe oryzae*, and *Rhizoctonia solani* (Ji *et al.*, 2008; Helene *et al.*, 2011; Prathuangwong *et al.*, 2012; 2013; Spence *et al.*, 2014; Montano, 2014; Homkrun and Kasem, 2018). The genus *Bacillus* has become

popular as biological control agents because of their antibacterial and antifungal peptide antibiotics activity (Sumi *et al.*, 2015; Kumar *et al.*, 2009). Heat and desiccation-resistant endospores are the attractive ability of genus because this is the essential to produce the bio-formulation (Chowdhury *et al.*, 2013). Using of secondary metabolites produced by *B. amyloliquefaciens* S20A1 cultured in optimized culture medium and secondary metabolites produced by S20A1 capable to be an alternative bio-bactericide for bacterial leaf blight disease control (Homkrun and Kasem, 2018).

Population density of antagonistic bacteria is an important factor that considers the pathogen protection, competition on to plant surface, leaf or root. The optimum cell density and growth stage are effect on bacterial survival and adaptation on plant part (Tampakaki *et al.*, 2009). Additionally, the high cost of culture media is a major challenge for mass production of biological control agents and there is a need to find alternative media added during the preparation of culture media. Selection of carbon and nitrogen sources as well as minerals supplements and C:N ratios are also major importance in the optimization of the medium nutrient supply (Yu *et al.*, 1998; Fisher, 1999; Carvalho *et al.*, 2010).

Generally, beans are well-known crop with highly nutrient contents which support the growth of bacteria. Locally available legume seeds such as green gram, black gram, cowpea and soybean are also used as alternative natural protein sources to formulate the culture media (Arulanantham *et al.*, 2012). Low cost of nutrient sources such as rice, corn, chickpea, cowpea, dhal, thinai, natural soy flour and processed soya flour were used to work as cost effective solid type media for the growth of some selected bacteria and fungi (Uthayasooriyana *et al.*, 2016). The aim of this study is to develop the budget-friendly modified media from locally available beans (black bean, mung bean and soybean) for the multiplication and biocontrol activities of *Bacillus amyloliquefaciens* S20A1, the beneficial antagonistic bacteria, to control bacterial leaf blight disease of rice caused by *Xoo*.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The plant pathogenic bacterial strains *Xoo* and the antagonist bacterium *B. amyloliquefaciens* S20A1 were obtained from the stock culture of Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand. The pathogenic and antagonistic bacteria were cultured on peptone sucrose agar medium (PSA) and yeast extract peptone agar medium (YPA), respectively.

Effect of General Culture Media on Cell Multiplication and Biocontrol Activities of *Bacillus amyloliquefaciens* S20A1

General culture media including Luria-Bertani broth (LB) (tryptone 10 g/L; yeast extract 5 g/L; NaCl 5 g/L), yeast extract peptone broth (YPB) (yeast extract 10 g/L; peptone 20 g/L), half of yeast extract peptone broth (Half YPB) (yeast extract 5 g/L; peptone 10 g/L) and nutrient broth (NB) (peptone 5 g/L; beef extract 3 g/L) were prepared in each 250 mL flask with 100 mL medium containing. The antagonistic bacterium was cultured in 5 mL nutrient broth (NB) for 18 hours by shaking 150 rpm at room temperature. The antagonistic bacterial *B. amyloliquefaciens* S20A1 cell suspension was adjusted to 0.2 optical density (O.D_{600nm}) using the spectrophotometer (Genesys 10S UV-Vis). The initial inoculum 500 µL of S20A1 was taken to transfer into above four media and incubated on rotary shaker maintaining 150 rpm at room temperature. The bacterial population in each medium broth was detected at 0, 12, 24, 36 and 48 hours by using serial dilution method on YP agar medium. This experiment was maintained with three replications.

The antibacterial activity of *B. amyloliquefaciens* S20A1 cultured in general culture media was tested by agar well diffusion method of Bauer *et al.* (1996). The inoculum of *Xoo* was cultured in peptone sucrose broth (PSB) for 36 hours and swabbed on the Petri dishes pouring with PSA medium. The supernatants of S20A1 from different culture media were collected at 48 hours by the Beckman Allgera X-14R refrigerated high speed centrifuge with 10,000 rpm for 10 min at 4°C. Then, the cell free filtrates were collected again by

passing the supernatants through the syringe filter that attached on sterile syringe. Thirty micro liter of each cell free filtrate was transferred into the 6 mm diameter agar well. The inhibition zone was observed 48 hours after incubation and this experiment was maintained with five replications.

The potential of S20A1 growth in general culture media for controlling disease was conducted under greenhouse conditions, the completely randomized design (CRD) was used with 5 replications. Jasmine Rice cultivar (KDML 105) which is the susceptible variety of *Xoo* was used in this study. The fourteen-days-old rice seedlings were transplanted into the plastic pots with commercial soil and kept in the greenhouse condition. After thirty days, rice plants were inoculated with *Xoo* bacterial suspension (10^8 CFU/mL) 10ml per each plant with three application times as pre-inoculation. Forty-eight hours after the last spray of each treatment, the bacterial suspension of *Xoo* (10^8 CFU/mL) was applied on tested rice plants by foliar spraying method of Reitama and Schure (1950) and maintained the optimum conditions for disease development. The commercial bactericide (Gentamicin sulfate 2%+ oxytetracycline hydrochloride 6% WP), distilled water and *Xoo* spray used as comparative control treatments. Totally twenty-five leaves from each treatment with five replications were randomly detected and measured the percentage of leaf infected area of *Xoo* by adobe image assessment with Adobe Photoshop CS 6 after 10 days disease inoculation. The percentage of disease incidence and t disease reduction were calculated comparing with non-treated control treatment by using the following formula (1) and (2) of Gnanamanickam *et al.* (1999).

$$\text{Disease incidence (\%)} = (\text{total lesion length/ total leaf length}) \times 100 \quad (1)$$

$$\text{Disease reduction (\%)} = [(\text{Disease incidence of } Xoo \text{ control (\%)} - \text{Disease incidence of treated plant (\%)}) / \text{Disease incidence of } Xoo \text{ control (\%)}] \times 100 \quad (2)$$

The cost of media per liter and their productivity were calculated based on their retailed price. The partial productivity of different culture media was modified the following productivity formula (3) followed as (Sumanth, 2000).

$$\text{Partial Productivity (CFU/ Baht)} = \text{Output (CFU/ mL)} / \text{Input (Baht /mL)} \quad (3)$$

Effects of Modified Bean Powder Extract on Cell Multiplication and Biocontrol Activities of *Bacillus amyloliquefaciens* S20A1

Different locally available beans including soybean, mung bean and black bean were firstly made as the powder condition using electric blender. Modified media were prepared as the following (Table 1). The media components were firstly mixed with water and boiled with constant stirring for 5 min. The bean powder extract solution was collected by passing through the double layer of muslin cloths to discharge the insoluble particles. Each 250 mL flask was filled with 100 mL of each media and autoclaved. The effects of these media on *B. amyloliquefaciens* S20A1 and their productivity were examined as the previous described test methods and measurements.

Table 1 The components ratio of different bean powder in modified media

No.	Media	Composition (g /L)
1.	Black bean (BB)	30
2.	Mung bean (MM)	30
3.	Soybean (SS)	30
4.	Black bean + Mung bean (BM)	15 + 15
5.	Black bean + Soybean (BS)	15 + 15
6.	Mung bean + Soybean (MS)	15 + 15

Potential of Modified Bean Powder Extract Media for Biocontrol Efficacy of Wettable Powder Formulation

Antagonistic bacterial inoculum *B. amyloliquefaciens* S20A1 was cultured in black bean+ soybean medium. After 36 hours of incubation period, the bacterial cell was precipitated by using refrigerator centrifuge at 10,000 rpm for 10 mins (4°C). Cell precipitate was adjusted with sterilized distilled water to get the 10^{13} CFU/ mL cell concentration and mixed with the sterilized carriers including talcum, kaolin and mixture of talcum + kaolin powders at the ratio of 15 mL of bacterial suspension per 100 g of carriers. The formulations were kept in the laminar flow for overnight under aseptic conditions to reduce the moisture content and then keep avoidance from direct sunlight. The preferred two bean powder extract media (black bean + soybean and mung bean+ soybean) were prepared and 0.5 g of each wettable powder formulation was used as starter inoculum that put into modified media and inoculated on 150 rpm rotary shaking at room temperature. After 24 hours incubation, the population of antagonistic bacterium was observed by serial dilution method and the biocontrol activities on rice bacterial leaf blight control was performed by post-inoculation method with five replications by CRD design. The prepared antagonistic bacterial suspensions supplemented were adjusted to 10^8 – 10^7 CFU/mL by diluted 10-folds water supplying. The tested plants were treated by 10 ml per each plant with three times foliar spraying with three days intervals. The percentage of infected area out of total leaf area was examined on 25 sample leaves collected from each treatment by using Adobe Photoshop CS6.

Statistical Analysis

The data was statistically analyzed by Duncan's test ($P = 0.05$) of SPSS software program version 22.

RESULTS AND DISCUSSION

Effect of General Culture Media on Cell Multiplication and Biocontrol Activities of *Bacillus amyloliquefaciens* S20A1

The mantagonistic bacterium *B. amyloliquefaciens* S20A1 in different general media of LB and YPB performed better than half of YPB and NB (Figure 1). As the trends of cell multiplication, incubation period at 36 hours was assumed as the suitable for the bacterial cell. The population density was not significantly different at $P = 0.05$ when observed in LB at 36 hours and 48 hours has 3.14×10^9 and 2.71×10^9 CFU/mL respectively whereas the YPB has 3.86×10^9 and 3.61×10^9 CFU/mL (Figure 1 and Table 2). Among these four media, the wider inhibition zone of 24.60 mm and 24.20 mm was measured in YP and LB respectively (Figure 2 and Table 2). Under greenhouse conditions, bacterial strain S20A1 cultured in YPB and LB showed the most effective disease reduction with 58.98% and 62.79% respectively when NB and half of HYB showed higher disease severity on tested plants. Being compared the cost per liter and log CFU per liter, LB was pointed out as the basal medium to get high results 6.2×10^{10} CFU/Baht with suitable price 49.9 Baht/L than 5.2×10^{10} CFU/Baht and 72.6 Baht/L for YP respectively (Table 2).

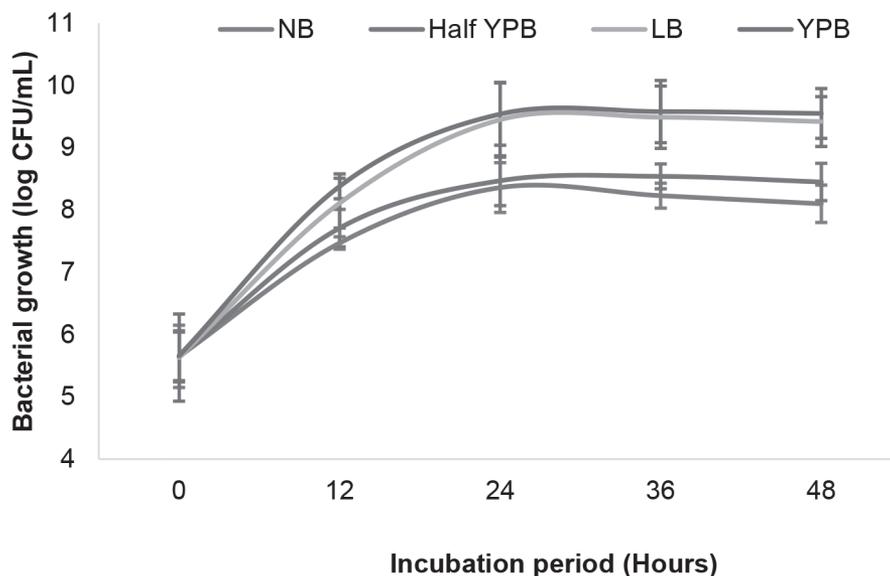


Figure 1 Colony forming unit (CFU) of *Bacillus amyloliquefaciens* S20A1 cultured in different culture media (Each point represents the mean of three independent experiments and error bars indicate \pm SE)

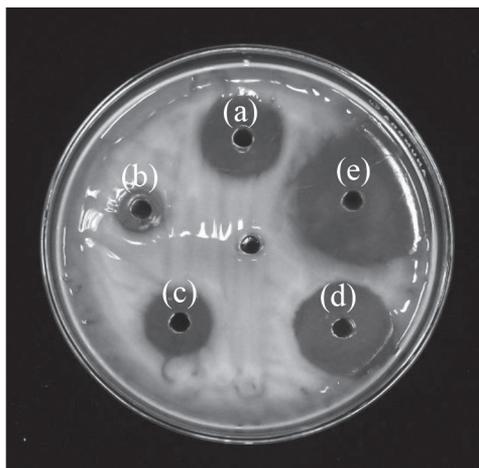


Figure 2 Activity of cell free filtrates collected from *B. amyloliquefaciens* S20A1 culturing in different culture media LB, Half YPB, NB and YPB (from (a) to (d)) were compared with commercial bactericide(e) to inhibit *Xanthomonas oryzae pv. oryzae* on PSA medium

Table 2 Effects of general culture media on cell multiplication and biocontrol activities of S20A1 comparing with their productivity

Media	Colony forming unit (CFU/mL) ¹	Inhibition zone diameter (mm) ²	Diseased incidence (%) ³	Disease reduction (%) ⁴	Cost of media per 1 L (Baht)	Partial Productivity (CFU/Baht)
NB	2.3 x 10 ⁸ ^b	15.80 ^d	34.40 ^b	46.92 ^d	20.38	1.1 x 10 ¹⁰
Half YPB	3.5 x 10 ⁸ ^b	23.00 ^c	29.65 ^{bc}	54.25 ^c	36.3	9.6 x 10 ⁹
YPB	3.8 x 10 ⁹ ^a	24.60 ^b	27.78 ^c	58.98 ^b	72.6	5.2 x 10 ¹⁰
LB	3.1 x 10 ⁹ ^a	24.20 ^b	24.12 ^c	62.79 ^b	49.9	6.2 x 10 ¹⁰
Bactericide ⁵	-	30.40 ^a	16.69 ^e	74.24 ^a	-	-
Non-treated ⁶	-	-	64.81 ^a	-	-	-
CV (%)	5.76%	2.94%	9.38%	5.61%	-	-
Mean ± S.E.	0.88	0.37	1.95	2.11	-	-

^{1,2,3,4} Means followed by the same letter in the same column were not significantly different at P = 0.05

⁵ Gentamicin sulfate 2% + oxytetracycline hydrochloride 6% WP

⁶ *Xanthomonas oryzae pv. oryzae* was only inoculated

As an antagonistic, the competition and antibiosis are the important mechanisms for the suppression of disease causing pathogenic microorganisms. Rhizosphere or phyllosphere BCAs generally protect the plant by rapid colonization, thus consuming completely the limited available substrates so that none is left for pathogens to grow (Pal and McSpadden Garden, 2011 credited to Lindsay, 1979). *Bacillus* species, well known as antibiotic producers, have special characteristics that make them good candidates as biological controls agents (Wulff *et al.*, 2002). *B. amyloliquefaciens* was versatile chemoorganotrophs like as *B. subtilis* therefore it can take the nutrient from fermentation of complex organic molecule. Nutrients components such as carbon and nitrogen are the basic nutrients for supporting energy sources and cell multiplication. Beef extract, peptone and yeast extract are the excellent nutrients using for the bacterial growth researches. Peptone contains almost all of amino acids and can accelerate the bacterial growth by combination of beef and yeast extract (Goto, 2012).

Homkrum and Kasem (2018) reported that yeast extract peptone medium was used as basal medium for the optimization of culture conditions in secondary metabolites production of *B. amyloliquefaciens* S20A1. In this present study, YPB and LB showed the best results to capable basal medium. After comparing the productivity, LB was selected because of its higher production rate in lower cost.

Effects of Modified Bean Powder Extract Media on Biocontrol Activities of *Bacillus amyloliquefaciens* S20A1

The bacterial growth of *B. amyloliquefaciens* S20A1 in modified media were performed as the previous trends and compared with LB (Figure 3). As the results of that the BS and MS media described as the best modified media than the other modified media for the cell multiplication 1.3 x 10¹⁰ and 1.0 x 10¹⁰ CFU/mL, inhibition diameter 30.60 mm and 30.00 mm, and disease reduction 63.10% and 63.82% respectively (Figure 4). The LB medium also showed the high performance in the reduction

of disease with 61.68%. They were not significantly different in controlling effect when comparing with the commercial bactericide at $P = 0.05$. The cost of ingredients per liter of the preferred media BS and MS media were 2.25 and 2.6 Baht/L whereas

the 5.8×10^{12} CFU/ Baht and 3.8×10^{12} CFU/ Baht respectively (Table 3). They provided a good source of low-cost raw materials for the microbial mass production and high productivity when comparing with the readymade LB medium.

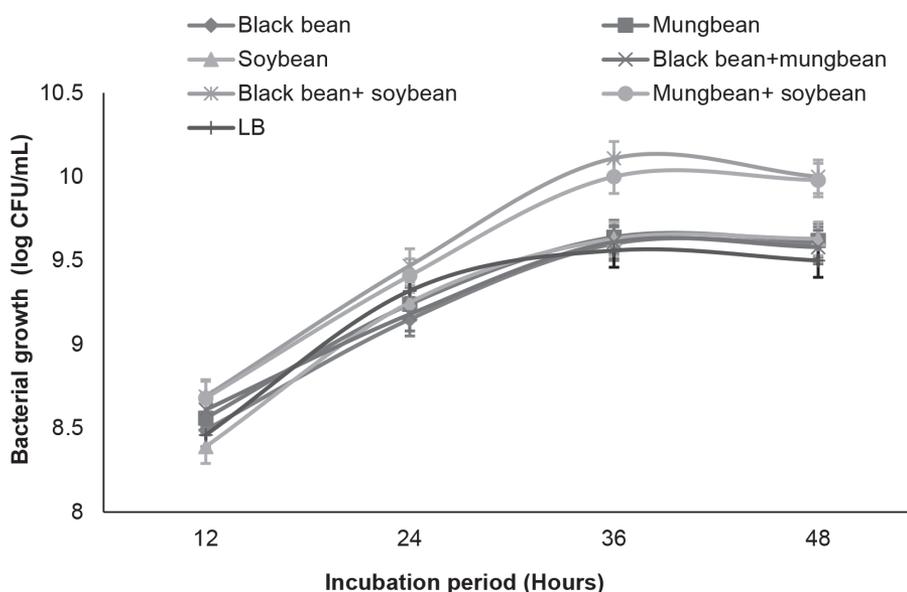


Figure 3 Colony forming unit (CFU) of antagonistic *Bacillus amyloliquefaciens* S20A1 cultured in bean powder extract media and LB medium (Error bars are standard error of the means)

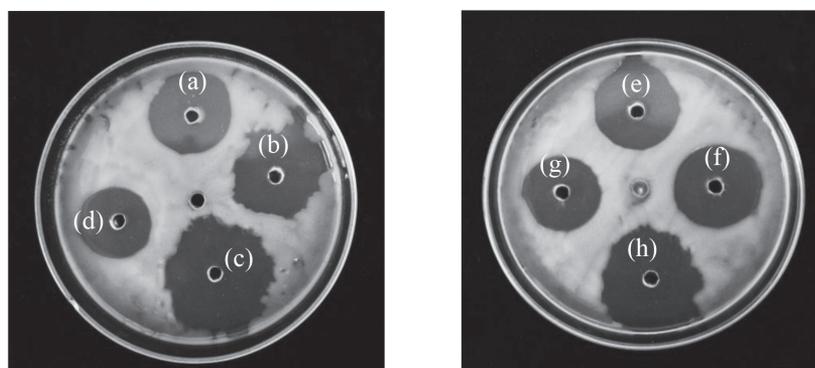


Figure 4 Activity of cell free filtrate collected from S20A1 cultured in different media black bean, mung bean + soybean, black bean + soybean, LB medium, mung bean, soybean (from (a) to (g)) respectively and commercial bactericide (gentamicin sulfate 2% + oxytetracycline hydrochloride 6% WP) (h)

Table 3 Effects of bean powder extract modified media on cell multiplication and biocontrol activities of S20A1 comparing with their productivity

Media	Colony forming unit (CFU/mL) ¹	Inhibition zone diameter (mm) ²	Diseased leaf area (%) ³	Disease reduction (%) ⁴	Cost of media per 1 L (Baht)	Partial Productivity (CFU /Baht)
LB	3.6 × 10 ⁹ d	24.00 ^d	21.06 ^a	61.68 ^a	49.9	7.2 × 10 ⁹
BB	4.4 × 10 ⁹ d	24.60 ^d	27.60 ^{bc}	49.78 ^{bc}	2.7	1.6 × 10 ¹²
MM	4.8 × 10 ⁹ cd	26.00 ^c	27.94 ^{bc}	49.16 ^{bc}	3.4	1.4 × 10 ¹²
SS	6.3 × 10 ⁹ c	27.40 ^b	23.16 ^{bc}	57.87 ^b	1.8	3.5 × 10 ¹²
BM	4.3 × 10 ⁹ d	24.40 ^d	29.31 ^b	46.67 ^c	3.05	1.4 × 10 ¹²
BS	1.3 × 10 ¹⁰ a	30.60 ^a	20.28 ^c	63.10 ^a	2.25	5.8 × 10 ¹²
MS	1.0 × 10 ¹⁰ b	30.00 ^a	19.89 ^c	63.82 ^a	2.6	3.8 × 10 ¹²
Bactericide ⁵	-	30.40 ^a	21.25 ^{bc}	61.33 ^a	-	-
Non-treated ⁶	-	-	54.96 ^a	-	-	-
CV (%)	8.92%	3.32%	15.41%	14.11%	-	-
Mean ± S.E.	0.48	0.57	2.66	5.06	-	-

^{1,2,3,4} Means followed by the same letter in the same column were not significantly different at P = 0.05

⁵ Gentamicin sulfate 2% + oxytetracycline hydrochloride 6% WP

⁶ *Xanthomonas oryzae* pv. *oryzae* was only inoculated

Deployment of different beans, soybean, mung bean and black bean, were applied because of their valuable nutrient contents basically as protein, amino acids and carbohydrates. Moreover, it still has glucose, at least 11 amino acids and phosphorous are important for the initiation of the various process of microbial metabolisms such as glycolysis, the pentose phosphate pathway and citric acid cycle (Hogg, 2005). The nutrients extraction method of these bean media was modified from the potato dextrose agar media (PDA) of Beever and Bollard (1969). As the diffusion theory, molecules can move from an area of higher concentration to an area of lower concentration. The rate of diffusion is depending on the size of molecules and speeded up by temperature. Therefore, the beans were powdered leading to break them down into the smaller size for saving cooking time and boiling with a temperature enhanced the solubility rate. Alfred *et al.* (1982) reported that 90% of sugar sucrose,

12% of nitrogen and about 60% of amino acids was extracted from California small white (CSW) by hot water diffusible method, the release components were also recovered in extract solution. The quality and concentration of C:N ratio can determine the bacterial growth and secondary metabolites production for antibacterial activities. Based on the USDA nutrient database, the estimation of C:N ratio of prepared black bean, mung bean and black bean + mung bean liquid media contained 3:1 ratio, soybean 1:1 ratio, and black bean + soybean and mung bean + soybean media 2:1 ratio. The 2:1 ratio supported the antagonistic bacteria S20A1 to perform the best growth (supplementary).

Preecha (2009) researched that the mass production and formulation of *B. amyloliquefaciens* KPS46 in liquid fermentation with the inexpensive nutrients soybean, fish meal and molasses, and organic cow carriers. They gave the growth equal to and better results at 48 hours 9.2 and 9.6 log

CFU/ml respectively than the nutrient glucose broth (9.3 CFU/ml). Arulanantham *et al.* (2012) carried out the study to find the feasibility of using legumes seeds supplying as nitrogen source in the growth of bacteria *E. coli*, *Bacillus* sp., *Klebsiella* sp., *Staphylococcus* sp. and *Pseudomonas* sp. Sartori *et al.* (2012) selected MSB medium (Molasses 20 g/l and Soybean powder 10 g/l) to support a rapid growth rate and high biomass production by comparing with four different media and survival after freeze-drying of *Bacillus amyloliquefaciens* and *Microbacterium oleovorans*.

Potential of Modified Media for Biocontrol Efficacy of Wettable Powder Formulation

The two preferred modified media were used for the multiplication of antagonistic bacterial S20A1 formulated in wettable powder carriers within the incubation period of 24 hours. In the continuous studies, the antimicrobial treatments were examined more than 50 percent of disease reduction was observed based on the non-treated plants (only *Xoo* inoculated). In the investigation of under greenhouse conditions, more than 50 percent of disease reduction was observed based on the non-treated plants. Treatment of Talcum + kaolin mixture formulation cultured in mung bean + soybean powder extract (MS) medium showed 65.56% the disease reduction percentage that non-significant different with 69.66% of the commercial antibiotic at $P = 0.05$ (Table 4).

Table 4 Potential of modified media on biocontrol activities of formulated S20A1 for bacterial leaf blight controlling under greenhouse conditions

Media	Formulation	Colony forming unit (CFU/mL) ¹	Diseased incidence (%) ²	Disease Reduction (%) ³
Black bean+ Soybean (BS)	Talcum	7.9×10^8	25.34 ^{de}	61.39 ^{bc}
	Kaolin	7.4×10^8	30.86 ^b	52.99 ^e
	Talcum + Kaolin	8.1×10^8	22.62 ^e	65.56 ^{ab}
Mung bean + Soybean (MS)	Talcum	7.8×10^8	28.82 ^{bc}	56.11 ^{de}
	Kaolin	7.5×10^8	27.37 ^{cd}	58.30 ^{cd}
	Talcum + Kaolin	8.8×10^8	23.14 ^e	64.80 ^b
LB	Fresh inoculum	7.1×10^8	23.18 ^e	64.68 ^b
Bactericide ⁴	-	-	19.92 ^f	69.66 ^a
Non-treated ⁵	-	-	65.65 ^a	-
CV (%)		9.19%	7.52%	5.19%
Mean \pm S.E.		5.84	1.27	2.02

¹ Colony forming unit of *Bacillus amyloliquefaciens* S20A1 after 24 hours incubation of antagonistic bacterial formulation cultured in modified media. Means followed in this column were not significantly different at $P = 0.05$

^{2,3} Means followed by the same letter in the same column were not significantly different at $P = 0.05$

⁴ Gentamicin sulfate 2% + oxytetracycline hydrochloride 6% WP

⁵ *Xanthomonas oryzae* pv. *oryzae* was only inoculated

Successful formulation based on the effective carriers, having the aim of preserving the organism and its antagonistic characteristics. Vermiculite, clay calcium sulfate and talcum are the most popular inorganic material for the formulation of antagonistic bacteria and fungi. Preecha (2009) reported that *Bacillus amyloliquefaciens* KPS46 survived in one selected formulation, talcum-based product at $8.4 \log_{10}$ CFU/g for 360-day storage at room temperature ($28 \pm 4^\circ\text{C}$) that declined to approximately 31.5% of original cell population. As pointed out by Anand (2010) that talc-based formulation of *Pseudomonas fluorescens* might increase the survival of bacteria and disease suppressed of chili anthracnose and powdery mildew. Jambhulkar and Sharma (2012) used the soybean bran, barley bran, wheat bran, talc powder and kaolinite powder for the bioformulation of *Pseudomonas fluorescens* to promote the rice seedling growth. Prathuangwong *et al.* (2013) investigated that two different carrier formulations of kaolin and talcum-based products to develop the *Pseudomonas fluorescens* SP007s biocontrol agent. Anith *et al.* (2014) also found that a cheap and farmer-friendly method for mass multiplication of formulated *Pseudomonas fluorescens* in boiled coconut water under non-sterile conditions. The culture media are able to multiply the biocontrol agent formulations without incurring additional cost. In that way, the cost of the input for the crop production can be reduced. The small amount of formulated bacterial inoculum was needed for mass multiplication. Media may enhance the population and activate the effects of formulated antagonistic bacterium.

CONCLUSIONS

Bacillus amyloliquefaciens S20A1 was an effective antagonistic bacterial strain to control the bacterial leaf blight disease of rice in Thailand. Among four general media, YPB and LB were observed the best media for cell multiplication, bacterial growth inhibition and under greenhouse conditions of disease control efficacy on bacterial leaf

blight of rice. Reasonably lower cost of production with greatest effects, LB medium was chosen as a representative conventional media for comparison to the modified media. Valuable nutrients contents of beans made as the good sources for the growth of antagonistic bacteria *B. amyloliquefaciens* S20A1. Bean powder mixture media of black bean + soybean medium (BS) and mung bean + soybean medium (MS) performed the best at cell multiplication and secondary metabolites production to inhibit the growth of *Xoo* comparing with the other modified media. The composition of 30 grams per liter at 1:1 ratio of each bean powder incubated on 150 rpm rotary shaker for 36 hours were the effective culture conditions for two modified media. Budget-friendly these two modified media reduced the cost of media per liter 22 times and 19 times comparing with LB medium. The modified media promoted cell population and activated the biocontrol efficacy of fresh inoculum but also formulated S20A1. Additional open field experiments would be needed to determine the efficacy and investment returns of *B. amyloliquefaciens* S20A1 bacterial strain under field conditions. This study was the pilot studies as the small-scale mass production of effective bacteria S20A1 for heading to industrial level.

ACKNOWLEDGEMENT

This work was supported by SEAMEO SEARCA (South East Asia Regional Center for Graduate Study and Research in Agriculture).

REFERENCES

- Alfred, C.O., M.G. Gregory, R.G. Micheal and R.W. Joseph. 1982. Nutrient composition of and digestive response to whole and extract dry beans. *J. Agri. Food Chem.* 30: 26–32.
- Anad, T., A. Chandrasekaran, S. Kuttalam, G. Senthilraja and R. Samiyappan. 2010. Integrated control of fruit rot and powdery mildew of chilli using the biocontrol agent *Pseudomonas fluorescens* and a chemical fungicide. *Biolo. Control.* 52(1): 1–7.
- Anith, A.N., V.G. Soumya, A. Sreekumar, R.A. Raj and N.V. Radhakrishnan. 2014. A cheap and farmer-friendly method for mass multiplication of *Pseudomonas fluorescens*. *J. Tro. Agri.* 52(2): 145–148.
- Arulanantham, R., S. Pathmanathan, N. Ravimannan and K. Niranjana. 2012. Alternative culture media for bacterial growth using different formulation of protein sources. *J. Nat. Prod. Plant Resour.* 2(6): 697–700.
- Bauer, A.W, W.M.M. Kirby, J.C. Sherris and M. Turck. 1996. Antibiotic susceptibility testing by standard single disk method. *Am. J. Clin. Pathol.* 45: 493–496.
- Beever, R.E. and E.G. Bollard. 1969. The nature of stimulation of fungal growth by potato extract. *J. Gen. Microbiol.* 60: 273–279.
- Carvalho, A.L.U., F.H.P.C., Oliveira, R.L.R. Mariano, E.R. Gouveia and A.M. Souto-Maior. 2010. Growth, sporulation and production of bioactive compounds by *Bacillus subtilis* R14. *Braz. Arch. Biol. Technol.* 53(3): 643–652.
- Chowdhury, S.P., K. Dietel, M. Rändler, M. Schmid, H. Junge, R. Borriss, A. Hartmann and R. Grosch. 2013. Effect of *Bacillus amyloliquefaciens* FZB42 on lettuce growth and health under pathogen pressure and its impact on the rhizosphere bacterial community. *PLOS ONE* 8(7): e68818.
- Cawoy, H., W., Bettiol., F. Patrick and M. Ongena. 2011. *Bacillus*-based biological control of plant diseases, Pesticides in the Modern World, In Pesticides Use and Management, Magarita Stoytcheva, Intech Open, DOI: 10.5772/17184. Available Source: <https://www.intechopen.com/books/pesticides-in-the-modern-world-pesticides-use-and-mangement/bacillus-based-biological-control-of-plant-diseases>.
- Fisher, S.H. 1999. Regulation of nitrogen metabolism in *Bacillus subtilis*: vive la difference!. *Mol. Microbiology.* 32(2): 223–232.
- Gnanamanickam, S.S., V.P. Brindha, N.N. Narayanan, P. Vasudevan and S. Kavitha. 1999. An overview of bacterial blight disease of rice and strategies for its management. *Current Science.* 77(11): 1435–1444.
- Goto, M. 1992 and 2012. *Fundamentals of Bacterial Plant Pathology*. Academic Press Inc. San Diego, California, USA.
- Hogg, S. 2005. *Essential Microbiology*. The University of Glamorgan. UK.
- Homkrun, P. and S. Kasem. 2018. Optimum conditions for secondary metabolites production and control efficacy on bacterial blight disease at rice of *Bacillus amyloliquefaciens* S20A1. *KMIJL Sci. and Tec. J.* 36(2): 22–32.

- Jambhulkar, P.P. and P. Sharma. 2012. Promotion of rice seedling growth characteristics by development and use of bioformulation of *Pseudomonas fluorescens*. Indian J. Agri. Sci. 83(2): 136–142.
- Ji, G.H., L.F. Wei, Y.Q. He, Y.P. Wu and X.H. Bai. 2008. Biological control of rice bacterial blight by *Lysobacter antibioticus* strain 13–1. Biolo. Control. 45: 288–296.
- Kumar, A., P. Saini and J.N. Shrivastava. 2009. Production of peptide antifungal antibiotic and biocontrol activity of *Bacillus subtilis*. Indian J. Exp. Biol. 47: 57–62.
- Lindsay, W.L. 1979. Chemical Equilibria in Soils. John Wiley & Sons. New York, U.S.A.
- Montano, P.F., C. Alias-Villegas, R.A. Bellogin, P. Del-Cerro, M.R. Espuny and I. Jimenez-Guerrero. 2014. Plant growth promotion in cereal and leguminous agricultural important plants: from microorganism capacities to crop production. Microbiol. Res. 169: 325–336.
- Pal, K.K. and B.M. Gardener. 2006. Biological control of plant pathogens. The Plant Health Instructor. DOI: 10.1094/PHI-A-2006-1117-02.
- Prathuangwong, S., D. Athinuwat, W. Chuaboon, T. Chatnaparat and N. Buensanteai. 2013. Bioformulation *Pseudomonas fluorescens* SP007s against dirty panicle disease of rice. Afr. J. Microbiol. Res. 7(47): 5274–5283.
- Prathuangwong, S., W. Chuaboon, T. Chatnaparat, L. Kladsuwan, M. Shoorin and S. Kasem. 2012. Induction of disease and drought resistance in rice by *Pseudomonas fluorescens* SP007s. CMU. J. Nat. Sci. 11(1): 45–55.
- Preecha, C. 2009. Formulation Development and Partial Characterization of Mechanism Conferred by *Bacillus amyloliquefaciens* KPS46 Against Soybean Disease. PhD thesis, Kasetsart University, Bangkok, Thailand.
- Reitsma, J. and P.S.J. Schure. 1950. “Kresek”, a bacterial blight disease of rice. Contribution of the Central Agricultural Research Station. Bogor. 117: 1–17.
- Sartori, M., A. Nesci and M. Etcheverry. 2012. Production of *Fusarium verticillioides* biocontrol agents, *Bacillus amyloliquefaciens* and *Microbacterium oleovorans*, using different growth media: evaluation of biomass and viability after freeze-drying. Food Additives and Contaminants : Part A : Chemistry, Analysis, Control, Exposure and Risk Assessment. 29(2): 287–292.
- Sonenshein, A.L., J.A. Hoch and R. Losick. 1993. *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology. Washington D.C., U.S.A.
- Spence, C.A., V. Raman, N.M. Donofrio and H.P. Bais. 2014. Global gene expression in rice blast pathogen *Magnaporthe oryzae* treated with a natural rice soil isolate. Planta. 239: 171–185.
- Sumanth, D.J. 2000. Total Productivity Management (TPmgt): A Systemic and Quantitative Approach to Complete in Quality, Price and Time. CRC press. Florida, U.S.A.
- Sumi, C.D., B.W. Yanga and Y.T. Hahm. 2015. Antimicrobial peptides of the genus *Bacillus*: a new era for antibiotics. Can. J. Microbiol. 61: 1–11.

- Tampakaki, A.P., E. Hatziloukas and N.J. Panopoulos. 2009. Plant Pathogen, Bacterial A2- Schaechter, Moselio, 655–677. Encyclopedia of Microbiology (Third Edition). Academic Press. Oxford. UK.
- USDA (United States Department of Agriculture). 2016. USDA National Nutrients Database for Standard Reference Legacy Release 28. Available source: <https://ndb.nal.usda.gov/ndb/search/list>. April 2018.
- Uthayasooryan, M., S. Pathmanathan, N. Ravimannan and S. Sathyaruban. 2016. Formulation of alternative culture media for bacterial and fungal growth. Der. Pharmacia letters. 8(1): 431–436.
- Wulff, E.G., C.M. Mguni, K. Mansfeld-Giese, J. Fels, M. Lubeck and J. Hockenhull. 2002. Biochemical and molecular characterization of *Bacillus amyloliquefaciens*, *B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris* pv. *campestris*. Plant Pathol. 51: 574–584.
- Yu, X., S.G. Hallett, J. Sheppard and A.K. Watson. 1998. Effects of carbon concentration and carbon-to-nitrogen ratio on growth, condiation, spore germination and efficacy of the potential bioherbicide *Colletotrichum coccodes*. J. Ind. Microbiol. Biotechnol. 20(6): 333–338.
- Xu, J., L. Zhou, V. Venturi, Y.W. He, M. Kojima, H. Sakakibari, M. Hofte and D. Vleeschauwer. 2015. Phytohormone-mediated interkingdom signaling shapes the outcome of rice- *Xanthomonas oryzae* pv. *oryzae* interactions. BMC Plant Biol. 15(10): 1–16.